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AN EFFICIENT DIRECT MULTIPLE SHOOT INDUCTION USING IMMATURE AND MATURE EMBRYOS OF INDIAN WHEAT CULTIVARS

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ABSTRACT

Wheat is the second most produced staple food crop in world. Callus mediated regeneration is time consuming and laborious. Efficient regeneration system via direct multiple shoots was developed for three cultivars (Lok-1, PBW-621 and HD-2967) of Indian wheat. MS medium supplemented with N6-benzylaminopurine (BAP) and thidiazuron (TDZ) with 20 different combinations were used for efficient multiple shoot induction. Immature and mature embryos were excised from seeds, vertically dissected into two pieces and used as explants. The MS medium containing 2 mg/l BAP and 2 mg/l TDZ was found to be the most effective for multiple shoot induction in all three cultivars. The highest number of shoots produced per explant was 13.3 ± 4.5 shoots of cultivar HD-2967. The other cultivars Lok-1 and PBW-621 produced 11.6 ± 1.5 and 11.6 ± 3.5 numbers of shoot, respectively. Elongated shoots were separated and successfully rooted on the MS medium containing 1 mg/l indole-3-acetic acid (IAA). The optimized media and protocol is independent of explants types (immature or mature), however the number of shoots produced per explant was observed to varying with cultivars. Hence, the present report shows an efficient direct multiple shoot induction and regeneration into plantlets of selected Indian wheat cultivars which would be useful resource for genetic transformation.

KEYWORDS: Wheat, Immature Embryo, Mature Embryo, In-Vitro Regeneration, Multiple Shoot Induction & Genetic Transformation

ABBREVIATIONS

DAA - day after anthesis; BAP - 6-benzylaminopurine; TDZ - thidiazuron; IBA - indole-3-butyric acid; IAA - indole-3-acetic acid

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Original Article

INTRODUCTION

Wheat (*Triticums aestivum*) is the second most produced staple food crop with the 735.02 million metric tons world production in year 2015/16. India has the largest growing area (31.47 million hectares) and is the second largest producer (86.53 million metric tons) in the world (http://www.fas.usda.gov/psdonline/circulars /production.pdf). Various biotic and abiotic stresses have been reported for negative impacts on the yield and nutritional quality of wheat. Genetic engineering opens opportunities to meet these challenges and allows introduction of novel desirable genes even across taxa.

However, efficient and rapid *in-vitro* regeneration is prerequisite for wheat improvement through genetic engineering and functional genomics approaches. Wheat is considered as a recalcitrant crop. Due to this nature, several explants of wheat have been used for their regeneration and transformation capacity, including the immature embryos (Cheng et al. 1997; Tao et al. 2011), mature embryos (Patnaik and Khurana 2003; Medvecká

and Harwood 2015), shoot apical meristem (Ahmad et al. 2002), scutellar tissue (Becker et al. 1994) microspores (Ziemienowicz et al. 2012), protoplasts (Ahmed and Sági 1993), inflorescence (Pareddy and Petolino 1990; Barro et al. 1999) and leaf base (Haliloglu 2006). Immature embryos and inflorescence are the most frequently used explants due to their higher regeneration efficiency (Ziemienowicz 2014). Callus-mediated regeneration using mature and immature embryos explants have been reported (Patnaik and Khurana 2003; Patnaik et al. 2006; Moghaieb et al. 2010; Yang et al. 2015). Most of *in-vitro* regeneration protocols for wheat are callus mediated using immature embryos, which is time consuming.

The direct regeneration of any plant species without intervening callus from any explant is a faster, time saving and ease to handle in various experiments (Rostami et al. 2013; Alok et al. 2016). Direct shoot induction and multiplication from explants have been reported in several cereals including rice (Nhut et al. 2000), maize (Pathi et al. 2013; Mushke et al. 2016) barley (Sharma et al. 2004; Ganeshan et al. 2006; Rostami et al. 2013), pearl millet (Arockiasamy et al. 2006) and oat (Ganeshan et al. 2006). Mature intact embryo from mature seeds of winter, spring and durum Canadian wheat cultivar was used for direct regeneration (Ganeshan et al. 2006). The regeneration and multiple shoot formation varies with different genotypes, it is critical to screen different varieties for efficient recovery of transgenic during genetic alteration (Rostami et al. 2013).

The main goal of the present report is to investigate the capacity of multiple shoot formation of Indian wheat cultivars (Lok-1, PBW-621 and HD-2967) using immature and mature embryos. It is desirable to optimize media and a protocol for efficient genotype independent regeneration of wheat in short-duration, which could be particularly useful for functional genomic analysis and trait improvement in wheat plants through genetic engineering.

MATERIALS AND METHODS

Explant Preparation

Immature seeds of three high yielding Indian wheat cultivars viz., PBW-621, LOK-1, and HD-2967 were collected from the plants grown at the research field of the National Agri-Food Biotechnology Institute (NABI). The individual plant spike of the each cultivar was tagged at the first day after anthesis (DAA) and the immature seeds were harvested at ~18 to 20 DAA. Immature seeds were stored at 4 ºC for two days, whereas mature seeds were imbibed for overnight and kept at 4 ºC. The seeds were surface sterilized in 0.1% aqueous $HgCl_2$ for 10 min with constant shaking and rinsed 4-5 times with autoclave Milli-Q water. Embryos were aseptically isolated and bisected into two pieces for the source of explant.

Media Composition and Preparation

MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 1 g/l enzymatic casein hydrolysate, 0.7 g/l L-proline, 7.98 µg/l copper sulphate, 100 mg/l *myo*-inositol and 30 g/l sucrose supplemented with different concentrations of thidiazuron (TDZ) and 6-benzylaminopurine (BAP). The combination of growth regulators and designation of media used for direct shoot induction and multiplication are mentioned in Table 1. The media were solidified with 0.8% agar. The pH was adjusted to 5.8 before autoclaving at 121 ºC at 15 lbs for 20 min.

Establishment of Cultures and Growth Condition

The isolated immature embryos were initially kept on basal MS medium for two days in low light and further vertically bisected into two parts. Approximately 60 explants of each of three cultivars were kept on each media.

The cultures were incubated at 22 ºC in the plant tissue culture chambers (Percival, USA) in low light intensity (40 µmol photon m^{-2} s^{-1}) for 4 weeks with 16/8-h light/dark. After 4 weeks, the emerged shoots were trimmed off from base and kept in low light for 2 weeks on their respective fresh medium. Further, the cultures were moved to 100 µmol photon m^{-2} s^{-1} for 4 weeks with 16/8-h light/dark. The experiment was performed in triplicate and number of emerged shoots per explants was recorded. A comparison between intact embryos and bisected embryo as initial explants was also noticed in term of shoots produced.

Rooting, Hardening and Acclimatization

The individual shoot was carefully separated from multiple shoot clumps and kept for rooting. Half strength MS medium, MS medium, MS medium containing 1 mg/l indole-3-butyric acid (IBA), and MS medium with1 mg/l indole-3-acetic acid (IAA) were tried for rooting response. The basic compositions of different media are presented in Table 2. The roots of plantlets were carefully washed to remove the agar gel and then transferred to pot containing Soilrite (Varsha Enterprises, Bengaluru). Each pot was covered with transparent polybag for acclimatization.

RESULTS & DISCUSSIONS

The number of shoots produced per explant (from immature bisected embryos) onto 20 different media of three (PBW-621, LOK-1, and HD-2967) cultivars was represented in Figure 1. The best media was MBT11 (2 mg/l BAP and 2 mg/l TDZ) which produced 11 to 13 shoots in all three cultivars within two months of incubation. The immature embryos were carefully isolated and kept on basal MS medium. Multiple shoots were induced on different media from intact immature (Figure 2a) and bisected immature embryos (Figure 2b). The two days old embryos emerged with coleoptiles and radical were bisected longitudinal as depicted by blue dash (Figure 2a). The response of bisected immature embryos into two parts was good with respect to intact embryos for rapid multiple shoots formation. Various attempts has been made for multiple shoot induction from embryo using different growth regulators in wheat, oat, barley and triticale (Ahmad et al. 2002; Ganeshan et al. 2006; Rostami et al. 2013). These reports were used intact embryo as initial explant whereas the present study reports two days pre-cultured bisected embryo that is responded efficiently for direct regeneration.

After first and second sub-culturing, the multiple shoot clumps generated from bisected immature/mature embryos were trimmed off from base as shown in figure by blue line (Figure 2c). The multiple shoot clumps after 8 weeks on MBT11 is shown in Figure 2d. We noted an average 11.6 ± 3.5, 13.3 ± 4.5, and 11.6 ± 1.5 number of shoots from each bisected immature embryo of cultivars PBW621, HD2967, and LOK1, respectively on MBT11 media (Figure 1). The MBT11 (2 mg/l BAP and 2 mg/l TDZ) media was also effective in case of mature embryos of all three cultivers. The regenration capacity due to addition of TDZ in media was positively corelated in monocot species (Shan et al. 2000; Ganeshan et al. 2006). Supplemetation of TDZ instead of BAP did not show any increase in the number of shoots in case of barley (Ganeshan et al. 2006; Rostami et al. 2013). Different combination of BAP and TDZ were tried for multiple shoot induction of durum wheat. Maximum 35 numbers of shoots per explant was observed when 4.5 µM of TDZ and 4.4 µM of BAP was supplemented on culture medium (Ganeshan et al. 2006). The MS medium supplemented with 3 mg/L BAP and 0.5 mg/L 2,4-D were used for multiple shoot formation in barley and it showed 12 shoots per explant (Rostami et al. 2013). We used 20 combinations of BAP and TDZ in media for optimzing maximum shoots per explants.

The individual shoots were carefully separated from multiple shoot clumps before placing on rooting media (Figure 2e). The best rooting response was observed when isolated individual shoot kept on MS medium supplemented with 1 mg/l IAA for 15 days (Figure 2f). The half MS and basal full strength MS medium did not show root induction even after 30 days incubation of explants. The individual dissected shoots were not survived when they were grown in MS media supplemented with 1 mg/l IBA. The rooted plantlets were carefully washed to remove the agar gel and then transferred to pot containing Soilrite and covered with polybags for one week (Figure 3a). All acclimatized plants were healthy and showed normal seed setting and maturation (Figure 3b).

CONCLUSIONS

In the present study a rapid, efficient and reproducible protcols by using immature and mature embryos were optimized for three Indian wheat varieties. The average number of direct regeneration of seven Iranian wheat cultivars reported with the range between 8.38 and 5.46 shoots per explant onto MS medium supplemeted with 2 mgl/1 2, 4-D and 10 mgl/1 BAP (Mokhtari et al. 2013). Most of callus mediated regeneration protocols in wheat using immature embryos take approximately 3-4 months. However, the present protocol is able to regenerate plantlets from immature as well mature embryos within 2-3 months. Such a short, genotypic independent and an efficient regeneration system for recalcitrant crop like wheat are ideally suited for genetic transformation research.

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Table 1: Different Combination of Growth Regulator in Media used for Direct Shoots Regeneration

Name of Medium	Plant Growth Regulator Combinations (mg/l)	
	BAP	TDZ
MBT1	0.5	0.5
MBT2	0.5	1
MBT3	0.5	2
MBT4	0.5	5
MBT5	1	0.5
MBT6	1	1
MBT7	1	2
MBT8	1	5
MBT9	2	0.5
MBT10	2	1
MBT11	2	2
MBT12	2	5
MBT13	5	0.5
MBT14	5	1
MBT15	5	2
MBT16	5	5
MBT17	-	0.5
MBT18	-	1
MBT19	-	2
MBT20	-	5

Table 2: Composition of Different Media

Name Of Media	Composition of Media
Basal MS	4.33 g/l Murashige and Skoog basal salts, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8 (NaOH)
Half MS	2.2 g/l Murashige and Skoog basal salts, B5 vitamins 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8 (NaOH)
MBT 1 to 20	4.33 g/l Murashige and Skoog basal salts, B5 vitamins, 1 g/l enzymatic casein hydrolysate, 0.7 g/l L-proline, 7.98 µg/l copper sulphate, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8 (NaOH) (Required concentration of BAP, TDZ or both were added before pouring the media)

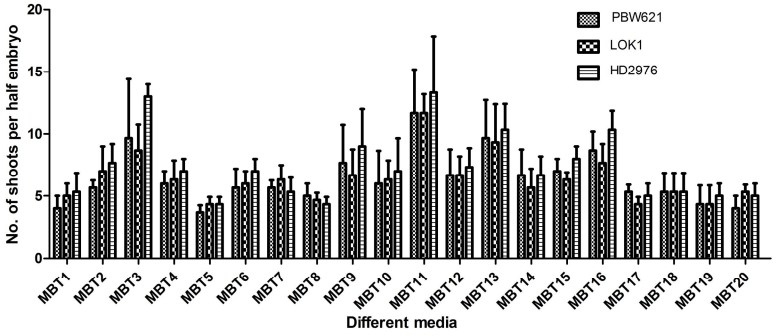

Figure 1: The Response of Different Media with Respect to Number of Shoots per Expalnt

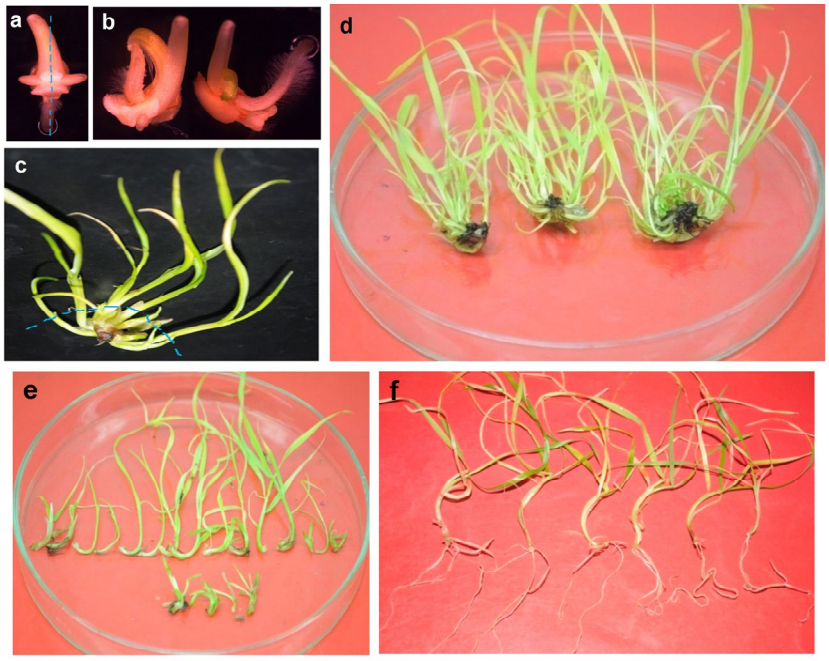

Figure 2: Different Stages of *In-Vitro* Direct Regeneration of Wheat. (a) 2 Days Old Pre-Cultured Intact Embryo, (b) Vertically Bisected Embryo Into Parts, (c) 3 Weeks Old Multiple Shoot Clump, (d) 6 Weeks Old Multiple Shoot Clumps, (e) Separated Individual Shoots, and (f) Rooted Plantlet on Rooting Medium

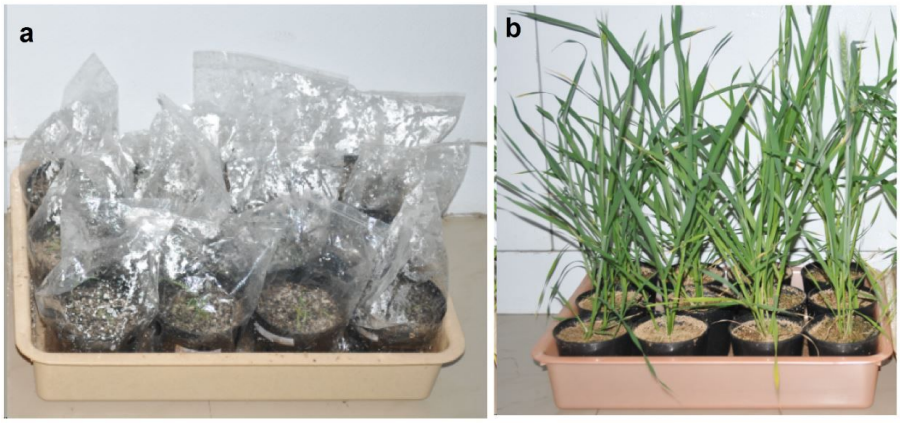

Figure 3: Hardening and Acclimatization of *In-Vitro* Rooted Plants. (a) Rooted Plantlets in Soilrite Containing Pots Cover with Transparent Polybags, and (b) One Month Old Acclimatized Healthy Wheat Plants